Technical limitations of the C1q single antigen bead assay to detect complement binding HLA-specific antibodies

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Author's contributions to work

CJT and SP conceived, designed and facilitated the study

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JM and GK compiled the result database

CJT, VK, DM and JAB undertook data analysis and interpretation

CJT and JAB jointly wrote the first draft of the manuscript

SP, VK and DM edited the manuscript and provided critical comment

All authors reviewed and agreed the final manuscript

Disclosure

The authors declare no conflicts of interest.

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Abbreviations:

DSA: donor HLA-specific antibody

EDTA: ethylene diamine tetraacetic acid

Fc: 'fragment crystallisable' portion of IgG heavy chain

IgG: immunoglobulin G

mAb: monoclonal antibody

MFI: mean fluorescence intensity

SAB: single antigen beads

Abstract:

Background: Solid-phase assays to distinguish complement binding from non-complement binding HLA-specific antibodies have been introduced, but technical limitations may compromise their interpretation. We have examined the extent to which C1q-binding to HLA-class I single-antigen-beads (SAB) is influenced by denatured HLA on SAB, antibody titre, and complement interference that causes a misleading low assessment of HLA-specific antibody levels.

Methods: Sera from 25 highly-sensitised patients were tested using Luminex IgG-SAB and C1q-SAB assays. Sera were tested undiluted, at 1:20 dilution to detect high-level IgG, and after EDTA treatment to obviate complement interference. Conformational HLA and denatured HLA protein levels on SAB were determined using W6/32 and HC-10 monoclonal antibodies respectively. Denatured HLA was expressed as HC-10 binding to untreated SAB as a percentage of maximal binding to acid treated SAB.

Results: For undiluted sera, Luminex mean fluorescence intensity (MFI) values for IgG-SAB and C1q-SAB correlated poorly (r^2 0.42). EDTA and serum dilution improved the correlation (r^2 0.57 and 0.77 respectively). Increasing levels of denatured HLA interfered with the detection of C1q binding. Consequently the correlation between IgG-SAB MFI and C1q-SAB MFI was lowest using undiluted sera and SAB with >30% denatured HLA (r^2 0.40) and highest using diluted sera and SAB with ≤30% denatured HLA (R^2 0.86).

Conclusion: Antibody level, complement interference and denatured HLA-class I on SAB may all affect the clinical interpretation of the C1q-SAB assay. The C1q-SAB assay represents a substantial additional cost for routine clinical use and we question its justification given the potential uncertainty about its interpretation.

Introduction

Over the last decade there have been major technological advances in the detection and characterisation of HLA-specific antibodies which in turn have enabled a better understanding of the role of alloantibodies in the rejection of kidney allografts [1-3]. Luminex single-antigen HLA-antibody detection bead (HLA-SAB) technology allows semi-quantitative detection of donor HLA-specific antibodies (DSA) with a high level of sensitivity and specificity [4]. Screening for the presence of DSA in the sera of potential recipients of a kidney allograft allows selection of suitable antibody-compatible donors and the development of *de novo* DSA together with the deposition of C4d on graft biopsy are diagnostic of antibody-mediated allograft rejection and associated with inferior long-term graft survival.

While the availability of HLA-SAB has revolutionised the detection of HLA-specific alloantibodies, it has become clear that not all patients with DSA identified by HLA-SAB experience allograft rejection and the time period between development of *de novo* DSA and deterioration in graft function can vary from months to many years [5]. The conventional HLA-SAB detects all IgG subclasses, irrespective of their ability to initiate complement activation. It has been suggested that those DSA that fix complement most effectively (IgG1 and IgG3) are those most likely to cause allograft injury and in an attempt to detect such DSA, the solid phase Luminex HLA-SAB assay has been modified to identify only those DSA that bind C1q, the first component of complement activation (C1q-SAB) [6-9]. In the C1q-SAB assay, the presence and level of complement fixing IgG isotypes bound to HLA-SAB are identified by the addition of exogenous C1q that bind the Fc region of complement fixing IgG and is detected using a fluorescent-conjugated anti-human C1q

antibody. Clinical application of the C1q-SAB assay has produced intriguing results that suggest C1q binding DSA are associated with graft damage whereas IgG-DSA that do not bind C1q are clinically benign [10]. It is not clear, however, whether this is due to the ability of the C1q-SAB assay to distinguish between complement fixing and non-complement fixing IgG subclasses or whether positivity may also be affected by HLA-specific antibody level and by technological artefacts that interfere with IgG and/or C1q binding [11-12].

The C1q-SAB assay represents a substantial additional cost for routine clinical use and we, and others, have questioned the justification for this given the potential uncertainty about its interpretation [13-14]. In the present study we show that, in addition to the influence of antibody levels and interfering factors (commonly referred to as the prozone phenomenon), the presence of denatured HLA class I protein on SAB may interfere with the C1q-SAB assay.

Materials and Methods

Study Group:

Sera were obtained from 25 highly sensitised patients awaiting deceased donor kidney transplantation at the Cambridge Transplant Unit (12 males: 13 females, median age 41 years, age range 20-61 years). All patients had calculated reaction frequency >85% (cRF) determined against a standardised panel of 10,000 consecutive UK organ donors [15] and had become sensitised by previous transplant alone (N=1), previous transplant plus blood transfusions and/or pregnancy (N=21), or blood transfusion plus pregnancy (N=3). Patient sera used in this study had been obtained and stored for the purposes of antibody screening and characterisation to guide selection of future antibody compatible donor and recipient pairs. This study was conducted in accordance with the UK Human Tissue Act that allows patients samples obtained for scheduled purposes (testing for HLA-specific antibodies) to be anonymised and used for pathology quality control and assay performance assessment.

Detection of IgG HLA specific antibodies and C1q binding using Luminex SAB:

Patient sera were tested undiluted (neat), after EDTA treatment and at 1 in 20 dilution using LabScreen^{TM} single-antigen HLA-class I specific antibody detection beads (One Lambda, Canoga Park, CA). The addition of EDTA to sera used in the SAB assay acts as a chelating agent that sequesters calcium ions and abrogates C1q formation, and obviates complement interference of FITC labelled IgG detection reagent. Addition of EDTA to sera results in only 5% dilution (5µL 6% EDTA solution to 95µL test serum) and in validation tests by ourselves and others, does not alter antibody binding to SAB [16]. In parallel, undiluted sera were tested using the C1QScreen^{TM} (One Lambda, Canoga Park, CA) according to standard

procedures. IgG-SAB and C1q-SAB binding levels were expressed as normalised mean fluorescence intensity (MFI) using HLA Fusion™ software (v3.2.0, One Lambda, Canoga Park, CA). All tests were undertaken at the same time using the same kit batches to minimise technical and operator variability.

Detection of conformationally folded and denatured HLA on SAB:

The level of conformationally folded (native) HLA class I protein expressed on the HLA-SAB was determined using W6/32 mouse monoclonal antibody (mAb) that recognises a monomorphic HLA class I epitope expressed upon association of heavy chain and beta2-microglobulin [17,18]. Denatured HLA protein expressed on SAB was determined using HC-10 mAb that was raised against free HLA class I heavy chain and loses reactivity upon association of heavy chain with beta2-microglobulin [19]. HC-10 shows heterogeneity in the level of binding to different class I free heavy chain and to account for this, denatured HLA was expressed as HC-10 MFI value obtained using untreated SAB as a percentage of maximal HC-10 MFI value obtained using denatured HLA class I following acid treatment of SAB. The purpose of the acid treatment of the HLA-SAB was to denature all the HLA protein and determine the maximal binding of HC-10 as shown in Supplementary Figure 1 [20].

Data analysis:

The correlation between IgG-SAB MFI (using undiluted, EDTA treated and diluted sera) and C1q-SAB MFI data was assessed using scatter plots in Microsoft Excel™ (Seattle, WA, USA). Pearson product moment correlation coefficients (r) were calculated to describe the proportion of variance between IgG-SAB MFI and C1q-SAB MFI. We predicted that the correlation would be a sigmoid curve, as turned out to be the case, because C1q binding will

remain negative until a threshold of IgG binding is reached that allows IgG bound to adjacent HLA molecules to be co-linked by C1q as described in reference 13. When this threshold is reached one would expect a linear correlation, until there are saturating amounts of IgG present, and therefore no additional binding of C1q. A polynomial trendline allowing three orders of data fluctuation (negative, increasing slope and saturation) was fitted by using the following equation to calculate the least squares fit through points: $y = b + c_1x + c_2x^2 + c_3x^3 + \ldots + c_6x^6$ where b and $c_1 \ldots c_6$ are constants (Microsoft Excel™, Seattle, WA, USA).

Results

Effect of EDTA treatment and dilution of patient test sera on IgG-SAB MFI and C1q-SAB MFI

Luminex HLA class I SAB assays were undertaken using undiluted sera, EDTA treated sera, and sera diluted 1 in 20. The results were compared with those obtained for unmodified sera tested using the C1q-SAB assay (Figure 1).

The correlation between IgG-SAB MFI using undiluted sera and C1q-SAB MFI (Fig. 1 panel A) was low (r^2 0.418) with many SAB populations showing high MFI values for IgG-SAB but low MFI for C1q-SAB and vice versa. This suggests that many sera contain high level IgG HLA class I specific antibodies that do not bind C1q, and low level IgG with strong C1q binding. The addition of EDTA to obviate complement interference in the IgG-SAB assay (Fig. 1 panel B) improved the correlation between the MFI values for IgG-SAB and C1q-SAB (r^2 0.568). Sera that previously displayed low level IgG binding but strong C1q-SAB binding were revealed by EDTA treatment to contain high level IgG-SAB binding. Following EDTA treatment sera still displayed high level IgG-SAB MFI against some antigen specificities that did not bind C1q-SAB. Dilution of test sera to identify only high titre HLA specific antibodies (Fig. 1 panel C) further improved the correlation between MFI values for IgG-SAB and C1q-SAB (r^2 0.769).

Analysis of conformationally folded and denatured HLA class I protein expression on single antigen beads

Supplementary Figure 1 shows the levels of conformationally folded (native) and of denatured HLA class I antigen (W6/32 and HC-10 mAb binding respectively) bound to the surface of HLA class I single antigen beads. The levels of native HLA class I antigen (W6/32

mAb binding) bound to the different bead populations was remarkably similar for all of the HLA-A and -B specificities and for most (13 of 16, 81%) HLA-C specificities. In contrast, the levels of denatured HLA class I antigen detected (HC-10 mAb binding) varied markedly between different bead populations and ranged between 19% and 91% (mean 69%, SD 21%) of maximal HC-10 binding on the beads (Figure 2). Nine of the 31 HLA-A specificities (29%) expressed low level (≤30%) denatured HLA antigen, while all 49 HLA-B and all 16 -C bead specificities expressed >30% denatured HLA.

Effect of denatured HLA protein on IgG-SAB MFI and C1q-SAB MFI

HLA-SAB populations were stratified according to the level of bound denatured HLA and the relationship with IgG-SAB MFI and C1q-SAB MFI was analysed (Figure 3). For HLA-SAB with >30% denatured HLA, the correlation coefficient between IgG-SAB MFI and C1q-SAB MFI was lower than that observed for SAB populations with ≤30% denatured HLA. This was the case for undiluted test sera (r^2 0.401 versus 0.647), EDTA treated sera (r^2 0.555 versus 0.721); and diluted sera (r^2 0.760 versus 0.861). These results indicate that denatured HLA on SAB can interfere with the C1q assay and hence lead to a poorer correlation between IgG-SAB MFI and C1q-SAB MFI: the difference was most marked using untreated and EDTA treated sera, and less notable using diluted sera. Of note, SAB specificities that displayed high level IgG-SAB MFI and low level C1q-SAB MFI were predominantly found in SAB populations with >30% denatured HLA, suggesting that denatured HLA protein interferes with the ability of IgG to bind C1q in this solid phase assay.

We next considered the effect of higher cut-off levels of denatured HLA class I protein expressed on SAB on the correlation between IgG-SAB MFI and C1q-SAB MFI (Table 1). As the cut-off level of denatured HLA was increased, the correlation between IgG-SAB MFI and

C1q-SAB MFI decreased progressively. This effect was more marked when undiluted test sera were used, than when diluted sera or sera treated with EDTA were used in the assays.

Relationship between IgG-SAB MFI and C1q-SAB MFI for individual patient sera

Analysis and display of sera from individual patients provided additional insights into the relationship between IgG-SAB MFI and C1q-SAB MFI. Individual patient sera showed two main patterns of HLA-SAB binding, as illustrated by results for the two selected patient sera shown in Figure 4. Using undiluted test serum, IgG-SAB MFI and C1q-SAB MFI for both patients correlated poorly (r^2 0.497 and 0.241 respectively). Following correction for the complement interference using EDTA treated sera and detection of high titre IgG antibodies using diluted sera, sera from patient 1 displayed a very good correlation between IgG-SAB MFI and C1q-SAB MFI (r^2 0.959 and 0.983 respectively). In contrast, use of EDTA and serum dilution of serum from patient 2 produced only a modest improvement in the correlation between IgG-SAB MFI and C1q-SAB MFI (r^2 0.720 and 0.819 respectively). When, for serum from patient 2, the effect of denatured HLA on C1q-SAB MFI was considered (Figure 5), the overall poor correlation observed for IgG-SAB MFI and C1q-SAB MFI was shown to be almost entirely restricted to SAB with >30% denatured HLA (r^2 undiluted sera 0.170; EDTA treated sera 0.593; diluted sera 0.719) and a very close correlation was seen for EDTA treated and diluted sera with ≤30% denatured HLA (r^2 EDTA treated 0.949; diluted sera 0.975).

Discussion

The findings from the present study highlight limitations in the use of solid phase bead assays to differentiate between complement binding and non-complement binding HLA specific antibodies in the serum of highly sensitised patients. The standard IgG-SAB assay has revolutionised HLA alloantibody screening, but it detects all IgG subclasses regardless of their ability to fix complement. It has been proposed that IgG antibodies that are complement binding are of greater clinical significance for predicting kidney transplant outcome [6-10, 21-25]. The introduction of the C1q-SAB assay as a tool to differentiate complement binding from non-complement binding IgG is therefore a very promising development.

While early studies using C1q-SAB suggested that detection of C1q binding DSA was independent of alloantibody level [10], no correction was made for interfering factors that cause a misleadingly low assessment of IgG-SAB binding [11-14,27-29]. The strong association of C1q and C3d donor specific antibodies detected using Luminex SAB assays with antibody mediated rejection and graft loss observed by Loupy [10] and Sicard [30] provides an important basis for prognostic monitoring and to guide treatment options. It remains to be shown, however, that *in vitro* detection of complement products in solid phase assays is independent of antibody strength [31]. The present analysis confirms that undiluted sera tested in the standard IgG-SAB assay gives results that correlate poorly with those obtained using the C1q-SAB assay, in keeping with the hypothesis that the two assays detect functionally distinct alloantibody populations. However, when antibody level and the contribution of complement interference are taken into account there is a clear correlation between the presence of high level IgG detected by IgG-SAB and the ability to bind C1q in

the C1q-SAB assay. This finding is in keeping with other recent studies questioning whether the C1q-SAB assay provides useful additional information [32]. There has been growing awareness that interfering factors [29,33,34] and antibody level [31,32] are important and the correlation between C1q-SAB binding and worse graft outcome may be an indication of antibody strength. In addition to C1q complement interference in HLA solid phase assays, Schwaiger et al (33) also demonstrated a role for complement split product, in particular C3d and C4d, as a cause of complement interference. Studies have shown that de novo formation of post-transplant donor-HLA-specific antibodies may be associated with C4d deposition in the capillaries of the graft and graft failure [reviewed in reference 35]. It is, however, likely that denatured HLA class I might pose similar limitations on other solid phase assay based complement detection techniques for C4d and C3d [30,35,36].

A limitation of the present study is that we did not investigate potential complement fixing and non-complement fixing IgG isotypes, although most sera contain a mixture of IgG isotypes and very few contain DSA with non-complement fixing IgG2 and IgG4 isotypes alone [11]. Otten et al reported that donor HLA-specific antibodies can co-recognize intact and denatured HLA [20]. They are present in the serum of patients in the absence of an allo-sensitisation event and may have high MFI, but are not associated with renal transplant rejection [20,37,38). The presence of antibodies to both denatured and native HLA on SAB could, therefore, give an artificially high MFI to bead specificities that carry large amounts of denatured HLA protein on their surface. Further, such antibodies might also bind C1q in the C1q-SAB assay, but have no clinical relevance following kidney transplantation.

Correction for the level of alloantibody alone in the IgG-SAB assay does not fully account for the differences obtained with IgG-SAB and C1q-SAB and there remain examples of high level

IgG binding that do not bind C1q. The novel aspect of the present study is the observation that the presence and level of denatured HLA protein on SAB may interfere with the ability of IgG to bind C1q in the solid phase assay. After correction for complement interference (using EDTA treated sera) and HLA-specific antibody titre (using diluted sera), and taking account of denatured HLA protein expressed on HLA-SAB, the correlation between results obtained using IgG-SAB and C1q-SAB was shown to be very good. This suggests that a positive IgG-SAB but negative C1q-SAB result does not necessarily indicate the presence of non-complement fixing antibodies, but instead, may indicate interference by denatured HLA in the C1q solid phase assay.

Antibody mediated activation of the classical pathway of complement is initiated when C1q binds the Fc region of antigen bound IgG. The relatively low affinity of the interaction between IgG-Fc and C1q is strengthened through the high avidity provided by the hexameric C1q molecule [39]. In physiological conditions, antigen present within a cell membrane are cross linked by IgG monomers to form antigen/antibody clusters that enable multivalent C1q to bind exposed Fc regions of multiple IgG molecules, giving a higher C1q binding constant. In contrast, in a solid phase assay, immobilised antigen bound to polystyrene beads is not able to form areas of high IgG/antigen clusters. In this situation, the ability of C1q to bind IgG-Fc is dependent on correct antigen/antibody spatial orientation that requires permissive spacing of antigen bound IgG-Fc to enable high avidity multivalent C1q binding [13]. Therefore, the combination of high SAB antigen density and high IgG levels are critical for C1q binding. In addition, the presence of denatured antigen on SAB interferes with native antigen spacing and may interfere with the ability of complement binding IgG isotypes to bind C1q.

In the present study, expression on SAB of high levels of conformationally folded HLA class I heavy chain/beta-2 microglobulin protein was, with the exception of three HLA-C locus antigens, remarkably consistent. This contrasts with early experience of Luminex HLA-SAB kits that showed overall lower and highly variable antigen expression on SAB populations [40]. It seems, however, that consistently high levels of native HLA class I protein expressed on most SAB populations has been achieved, in some cases, at the expense of high levels of denatured (free class I heavy chain) HLA protein, and this is most notable for HLA-B and HLA-C [20]. It may be that the manufacturing process to increase the levels of HLA protein has contributed to the variable levels of denatured protein or that some HLA specificities are more stable than others. The absolute percentages of HC-10 binding (before and after acid treatment) shown in Figure 2 are likely to be influenced by (a) the extent to which HC-10 binds the given HLA class I specificity (because there is published evidence that HC-10 binds different specificities to a variable extent), and (b) the 'non-specific' effects of acid treatment on antigen, which means the percentage of binding before acid treatment and as a percentage of total binding after acid treatment are likely to be proportionate.

It has been reported by Tran et al [41] that W6/32 can bind both complete HLA molecules and free HLA-B heavy chains, although the absence of W6/32 binding free heavy chain expressed on acid treated SAB clearly demonstrates that W6/32 does not bind to a significant extent to denatured HLA class I protein (all MFI <150) [20]. Whilst expression of denatured HLA will have little or no effect on IgG binding to native HLA proteins in the conventional IgG-SAB assay, our data suggests that high levels of denatured HLA causes technical interference in the C1q-SAB assay that can produce misleading results. Our data indicates that the effect of increasing levels of denatured HLA on SAB is most notable using

untreated sera, but there is little effect using diluted sera. The likely explanation for the less pronounced correlation is that when sera are diluted, only high titre HLA-specific antibodies are detected by binding to SAB, and the ability of these to bind C1q is less influenced by the presence of denatured HLA on SAB.

It should also be remembered that the patient cohort in our present study were particularly highly sensitised and none were sensitised by blood transfusion alone. It is not, therefore, clear if our findings are also applicable to patients with lower levels of sensitisation (e.g. by blood transfusion alone), but the ability of HLA antibodies to bind C1q is likely to relate to antibody titre and affinity and not the nature of the alloantibody priming event per se. Further, the thrust of our study was to highlight the technical limitations of the C1q-SAB and we did not therefore seek to correlate such data with biopsy findings and clinical outcomes which was beyond the scope of this study.

Given our findings with respect to HLA class I, analysis of denatured HLA class II on C1q binding would now be of interest, but again was beyond the scope of the present study. Although there are reports of suspected antibody binding to denatured HLA class II molecules in Luminex SAB assays, there is no direct evidence that antibody binding is to denatured protein [42,43]. A potential problem of undertaking a similar analysis for HLA class II is that we are unaware of comparable mAbs to HC-10 that detect denatured HLA class II. To undertake such a study would ideally require analysis of free alpha and beta chains of HLA-DR, -DQ and -DP and we are not aware that appropriate reagents are available to perform such a study.

In conclusion, the present study shows that antibody level, interfering factors and the presence of denatured HLA protein on class I SAB may all affect the clinical interpretation of

the C1q assay. Interest in the C1q binding assay to detect clinically relevant HLA antibodies continues to increase and our study highlights the importance of technical factors that may affect its clinical interpretation. Given these uncertainties and the considerable costs involved, the addition of the C1q-SAB assay into routine clinical practice is difficult to justify.

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Table 1.

Effect of denatured HLA protein level expressed on HLA class I SAB and the relationship between IgG-SAB MFI and C1q-SAB MFI

% denatured HLA expressed on SAB populations	No. of SAB combinations	Untreated serum (r)	EDTA treated serum (r)	Diluted serum (r)
All SAB	2400	0.418	0.568	0.769
≤30% denatured SAB	225	0.647	0.721	0.861
≤40% denatured SAB	475	0.549	0.670	0.826
≤50% denatured SAB	500	0.544	0.665	0.821
≤60% denatured SAB	625	0.501	0.629	0.823
≤70% denatured SAB	923	0.454	0.582	0.802
≤80% denatured SAB	1416	0.417	0.560	0.792

^ Pearson's Correlation Coefficient

Figure legends

Figure 1: *Effect of serum treatment on IgG-SAB and C1q-SAB binding:*

Undiluted sera (panel A), EDTA treated sera (panel B) and 1 in 20 diluted sera (panel C) obtained from 25 highly sensitised patients were tested using Luminex HLA class I IgG-SAB. The results (IgG-SAB MFI, x-axis) were compared to that obtained for undiluted sera tested using C1QScreen^{TM} (C1q-SAB MFI, y-axis). The results show improved correlation coefficients (r^2) between IgG-SAB MFI and C1q-SAB MFI following correction for the prozone effect (EDTA treated sera) and taking account of high titre IgG HLA specific antibody (diluted sera) compared to the conventional assay performed using untreated sera.

Figure 2

Assessment of levels of denatured HLA class I protein on single antigen bead populations: The level of denatured HLA class I protein expressed on SAB was determined by comparing HC-10 MFI value for each SAB population tested using untreated SAB as a percentage of maximum HC-10 MFI value tested using acid treated (denatured) SAB.

Figure 3: *Effect of denatured HLA class I protein expression on IgG-SAB and C1q-SAB binding:*

Undiluted sera (panels A and B), EDTA treated sera (panel C and D) and 1 in 20 diluted sera (panels E and F) were tested using Luminex HLA class I IgG-SAB. The results (IgG-SAB MFI, x-axis) were compared to that obtained for undiluted sera tested using C1QScreen^{TM} (C1q-SAB MFI, y-axis). SAB populations were stratified into two groups according to ≤10% denatured

HLA protein expression (panels A, C and E) and >10% denatured protein (panels B, D and F). The results show that for each serum treatment, the correlation coefficient between IgG-SAB MFI and C1q-SAB MFI was higher for SAB populations that express low levels (≤10%) of denatured HLA compared to high levels (>10%) of denatured HLA.

Figure 4: *Examples of IgG-SAB and C1q-SAB MFI binding profiles for two selected patients:* Undiluted sera (panels A and B), EDTA treated sera (panel C and D) and sera diluted 1 in 20 (panel E and F) obtained from two selected patients (Patient 1 and Patient 2) were tested using Luminex HLA class I IgG-SAB. MFI values for IgG-SAB (x-axis) were compared to that obtained for undiluted sera tested using C1QScreen^{TM} (C1q-SAB, y-axis). For Patient 1 untreated serum the correlation coefficient (r^2) between MFI values obtained using IgG-SAB MFI and C1q-SAB was poor, but following correction for the prozone effect (EDTA treated serum) and taking account of high level IgG (diluted serum) the correlation was near perfect. For Patient 2, the correlation between IgG-SAB MFI and C1q-SAB MFI using untreated serum was poor and EDTA treatment and serum dilution gave only a relatively modest improvement.

Figure 5: *Effect of denatured HLA class I protein expression on IgG-SAB and C1q-SAB binding profiles for patient 2:*

For patient 2 serum, MFI values obtained using IgG-SAB (x-axis) were compared to those tested using C1QScreen^{TM} (C1q-SAB, y-axis). The results were stratified according to the level of denatured HLA (≤10% versus >10%) expressed on the different SAB populations. For SAB that expressed ≤10% denatured HLA there was a high correlation between MFI values obtained using IgG-SAB and C1q-SAB assays, particularly when using EDTA treated and diluted serum. In contrast, for SAB that expressed >10% denatured HLA, for undiluted serum

the correlation was very poor, and showed only a modest improvement using EDTA treated and diluted serum.

Supplementary Figure 1 legend

Analysis of conformational folded and denatured HLA class I on single antigen beads: W6/32 is a mouse mAb that binds a monomorphic epitope on conformationally folded HLA class I protein (HLA class I heavy chain in non-covalent association with beta2-microglobulin) and does not bind free HLA class I heavy chain. HC-10 is a mouse mAb that binds β2-microglobulin free (denatured) HLA class I heavy chain. mAb binding to untreated and acid treated (denatured) HLA class I SAB was performed as described by Otten et al. [20]. The results show consistent high levels of conformationally folded HLA class I protein expressed on all but three SAB populations, but highly variable levels of HC-10 binding (Supplementary Figure 1a). W6/32 binding to free HLA class I heavy chain was abrogated following acid treatment of SAB, but HC-10 binding increased (Supplementary Figure 1b).